Production of Chicken Egg Yolk Antibody (IgY) Against Recombinant Cholera Toxin B Subunit and Evaluation of Its Prophylaxis Potency in Mice

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ABSTRACT

Background: Cholera toxin (CT), responsible for the harmful effects of cholera infection, is made up of one A subunit (enzymatic), and five B subunits (cell binding). The release of cholera toxin is the main reason for the debilitating loss of intestinal fluid. Inhibition of the B subunit (CTB) may block CT activity. **Objective:** To determine the effect of anti CTB-IgY against oral challenge with *V. cholera* in suckling infant mice. **Methods:** The binding domain of cholera toxin was amplified and ligated into pET28a vector. The pET28a (+)/ctb expression vector was confirmed by endonuclease digestion and sequence analysis. The expression of recombinant CTB in *E. coli* was performed by induction with IPTG. After immunizing the chickens with recombinant CTB, IgY was purified by water dilution method and NaCl precipitation and analyzed by SDS-PAGE. Moreover, the activity and specificity of the IgY antibody were assessed by ELISA. **Results:** The SDS-PAGE and western blot techniques showed that CTB protein was successfully expressed and specifically recognized by polyclonal antibodies against the cholera toxin. The oral administration of anti- (*V. cholera*+CTB) in infant mice in challenge with active *V. cholera* bacterium demonstrated high rate of survival. **Conclusion:** The increase in the number of antibiotic resistant bacteria implies the necessity of finding novel antibiotics. Our results suggest the possibility of passive protection from purified IgY, hence implying that anti CTB-IgY may be useful in the treatment of cholera infections.

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Keywords: CTB, IgY Prophylaxis, Recombinant Protein, *Vibrio cholerae*

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INTRODUCTION

As an infectious disease caused by the bacterium *Vibrio cholera*, cholera affects the water transport in the small intestine. The pathogenesis of this organism is due to a secreted toxin, namely cholera toxin (CT), which causes the infected host to hyper-secrete electrolytes and water, leading to dehydration and ultimately death by diarrhea. CT is the main virulence factor of the pathogen *Vibrio cholera* (1,2). The cholera toxin has two main subunits: Subunit A (for toxic activity) and subunit B (for binding). Subunit A (240 amino acids; MW 28 kD) contains two non-identical domains, A1 (22 kD) and A2 (5 kD). Subunit B (~56 kD) is composed of five identical, non-covalently bound polypeptide chains (103 amino acids; MW 11 kD), arranged in a ring-like pentameric configuration (3). After being secreted from the *Vibrio cholera*, CT binds to the enterocytes (intestinal cells) by the interaction between the subunit B and GM1 ganglioside receptor on the enterocytes, which subsequently promotes the toxin endocytosis. Next, A1 turns into an active enzyme after separating from the A2 domain. After that, the A1 domain of subunit A enters the cytosol and activates the adenylate cyclase to produce cAMP through G proteins, triggering the activation of cystic fibrosis transmembrane conductance regulator (CFTR), leading to a severe aqueous diarrhea caused by cholera (4). Through the use of *Escherichia coli* (5,6), *Lactobacillus* (7), *Bacillus subtilis* (8) and *Vibrio cholera*, certain recombinant systems have been developed for the expression of recombinant subunit B of cholera toxin (rCTB) (9). Cholera toxin B subunit (CTB) has been shown to be expressed in transgenic tobacco (10) and rice (11,12) plants. The CBT gene expression has been investigated in several bacterial, yeast and plant systems by other researchers. Passive antibody prophylaxis is a method used to protect patients against infectious diseases. Primarily developed in the twentieth century, many immunoglobulin preparations are currently being employed in clinical use. Given the need for new antimicrobial treatments and advances in the field of antibody, the application of antibody-based therapy on prophylaxis and the treatment of infectious diseases have been the subject of many studies (13). Egg yolk-derived immunoglobulins (IgY) are recognized as excellent sources of polyclonal antibodies, because in compared with mammalian antibodies, they are more facile to deal with and less expensive. Furthermore, this method is noninvasive and obtained a large-scale production over other antibodies (14). Egg yolk immunoglobulin has been used for its therapeutic features like protection against pathogen infections, and the prevention against or control of intestinal infections, such as rotavirus (15,16), enterotoxigenic *E.coli* (17), *Salmonella* (18,19), *Helicobacter pylori* (20,21), and exhibited good efficiencies. These studies, in general, have proven the potential advantage of IgY with regards to CTB *Vibrio cholera* in order for controlling and preventing Cholera disease (22,23). The objective of the present study was to produce cholera toxin B subunit in *E. coli*, and egg yolk antibody (IgY) against the recombinant cholera toxin B subunit.

MATERIALS AND METHODS

DNA Cloning of the CTB Gene. The genomic DNA *Vibrio cholera* was extracted by the standard CTAB method (24). The full length of CTB (312 nt of ctb + 20 nt of Restriction enzymes) was amplified by polymerase chain reaction (PCR). The specific primers (F 5'TGCAGAATTCACACCTCAAAATATTACTG3'; R 5' TATCAAGCTTT

TAATTTGCCATACTAATTGC3') containing EcoRI and HindIII sites, were designed using Oligo 6 software according to CTB sequences of *Vibrio cholera* reference strain (GenBank AccessionNC_015209); these primers were later synthesized. The gene PCR process was carried out in a mixture composed of 200 ng of DNA, 10 pM of forward and reverse primers, 0.4 mM of dNTP mix, 3 mM of $MgCl_2$, 1 XPCR Buffer and 2 U Taq DNA polymerase (Cinnagen Tehran, Iran) in 25 µl on a thermal cycler (Techne Gradient Staffordshire, UK). The procedure was continued in the following path: Initial denaturation at 94°C for 5 min and 30 cycles of 45s at 94°C, 30s at 58°C, 45s at 72°C, and a final 5 min at 72°C. The size of amplified DNA fragment was analyzed by electrophoresis on 1% (w/v) agarose gel; the fragments were digested with restriction enzymes (EcoRI and HindIII) and purified by purification kit (Bioneer Daejeon, Korea). The pET28a plasmid was isolated from *E. coli* DH5α by use of alkaline lysis method. The pET28a was digested with the same restriction endonucleases. Further measured were the concentrations of the purified and digested amplified fragment and pET28a plasmid. Subsequently, employing 3:1 molar ratio of the fragment inserted into vectors, ligation reaction was accomplished via T4 DNA ligase following 18 h at 14°C; the amplified fragment was ligated into the pET28a vector. These vectors were transformed into *E. coli* BL21 (DE3) competent cells using heat shock method. The transformed cells were grown in LB medium supplemented with kanamycin (80 µg/ml). The selected clones were analyzed by (EcoRI and HindIII) restriction enzymes and sequence analysis.

Expression of CTB in *E. coli*. The pET28a-ctb recombinant plasmid was transformed into *E. coli* BL21 (DE3). The colonies were inoculated into the LB medium containing kanamycin with a concentration of 80 µg/ml and grown at 37ºC overnight. 100 µl of cells were then transferred into 5 ml of LB medium and grown at 37ºC with 150 rpm until OD600 0.6 was reached. The recombinant protein was expressed through inducing with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 37ºC for 5 h. The cells were harvested by centrifugation at 3000 ×g for 5 min. Pellets were resuspended in lysis buffer with a pH of 8.0, containing 100 mM NaH_2PO_4, 8 M Urea, and 10 mM Tris-HCl. The expression was analyzed by 12% SDS-PAGE.

Purification of the Recombinant CTB Protein. Protein was purified using Ni–NTA affinity chromatography (Qiagen) under denaturation condition. The cell pellet harvested from 50 ml of the grown cell was resuspended in 2 ml lysis buffer and disrupted by sonication for 6 times (30 sec, 70 Amp/0.5 cycle) under cold conditions; following centrifugation, the supernatant was discarded. Ni–NTA resin equilibrated with the lysis buffer and supernatant was loaded on column. The column was washed with washing buffer (100 mM NaH_2PO_4, 8 M Urea, 10 mM Tris-Cl. pH=6.3) and the bound protein was eluted with an elution buffer (100 mM NaH_2PO_4, 8 M Urea, 10 mM Tris-Cl. pH=4.5). Fractions were analyzed by 12% SDS-PAGE. The eluted proteins were refolded in a step wise dialysis under reducing conditions from 8 M to 6 M, 4 M to 2 M and finally 0 M urea in phosphate saline buffer (PBS).

Western Blotting. The CTB separated by 12% SDS-PAGE was prepared according to the Laemmli protocol with modifications in accordance with Ladner (25). The CTB in the gel were transferred to PVDF blotting membrane in a semi-dry blot procedure. The membrane was blocked by 1% skim milk in the PBS. Once blocked, the membrane was washed three times with PBS for 10 min then incubated with 1:2000 dilutions of Anti-CTB in PBST (0.1% Tween 20) for 1.5 hours. Once again, the membrane was washed three times for 15 min with PBST; subsequently, goat anti-mouse IgG antibody (1:2000

in PBST) and the secondary antibody were added to the membrane and incubated for 30 min at 37ºC. The membrane was then washed four times and developed with DAB reagent and to stop the chromogenic reaction, the membrane was rinsed twice with distilled water.

Immunization. 20-week-old hens were immunized in order to obtain CTB-specific IgY loaded eggs. Hens were kept in individual cages with food and fresh water. They were immunized subcutaneously at the breast region with CTB protein mixed with complete Freund's adjuvant. For immunization, 200 µg of CTB protein were emulsified with an equal volume of complete Freund's adjuvant. The hens were injected with the recombinant CTB protein two and four weeks following the initial immunization; it should be noted, however, that the CTB was emulsified with incomplete Freund's adjuvant. Two hens were kept non-immunized with PBS injection only. Blood samples were taken from the vena basilica of the hens at 7-day intervals, eggs were collected daily, marked and stored at 4ºC (26).

IgY Antibody Purification. Egg yolk was separated from the white part and then rolled on paper towels to remove the adhering egg white. The yolk membrane was ripped and collected without the membrane. The yolk samples were mixed with six volumes of cold acidified water (pH 5.0 adjusted with 0.5 M HCl). The mixture was frozen at -20°C for 1 h. After thawing at room temperature, it was centrifuged at 13500 ×g at 4°C for 15 min; the supernatant was then collected and filtered through a Whatman filter. Then NaCl was added to 2 M concentration that adjusted at pH 4.0 and 0.5 M HCl, The solution was kept at room temperature for 2 h and was centrifuged at 3700 ×g and 4°C for 20 min and dissolved in phosphate-buffered saline. The obtained IgY protein was dialyzed and its concentration estimated for protein content by Bradford method. The purity of IgY was monitored by the sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (27).

ELISA. The titer of the antibodies generated against CTB was specified by indirect enzyme-linked immunosorbent assay (ELISA). Ninety-six-well plates were coated with CTB (5 µg/well). After blocking with 5% skim milk, plates were subsequently washed and incubated with sera (diluted in PBS, 100 µl/well). They were then washed with phosphate-buffered saline -Tween (0.05% Tween 20 in PBS [pH 7.2]) and incubated for 1 h following the addition of alkaline phosphatase conjugated rabbit anti-chicken IgY (Promega). The plates were washed with PBS-Tween, and disodium p-nitrophenyl phosphate was added as a substrate to each well. After incubation for 10 min, the reaction was stopped by the addition of 2.5 M H_2SO_4. The absorbance was measured at 492 nm using a microplate reader.

Animal Challenges. The infant mouse challenges were performed as previously described (28,29). All pups used in the experiments were 6 days old and weighed 3.5 ± 1.0 g Infant mice were isolated from their mothers and challenged orally with 50 µl bacterial suspension. *V. cholera* was grown in LB medium until the optical density reach between 1.0 and 1.5 at 600 nm, then harvested by centrifugation, and suspended in sterile PBS. The inoculum contained between 4×10^8 and 1×10^9 bacteria, as was determined through colony counts. -2, -1, 0, 1 and 2 h after this inoculation, the infant mice were administered 50 µl of each (1 mg/ml) anti CTB-IgY. The infant mice were monitored for survival over the course of 48 h. Table 1 illustrates the representative data at 48 h post challenge.

Statistical Analysis. Statistical analyses were carried out by SPSS 21.0. (Inc, Chicago, IL,USA) Comparisons among groups were performed by One-way analysis of variance ANOVA test. For comparison between two groups, Student's t-test was applied. P-value less than 0.05 were considered significant.

RESULTS

Expression of the recombinant CTB protein.
The coding region (312 nt of ctb + 20 nt of restriction enzymes) of the CTB was amplified by PCR (Figure 1) and inserted into pET28a plasmid. The recombinant clones were confirmed by restriction digestion (Figure 2).

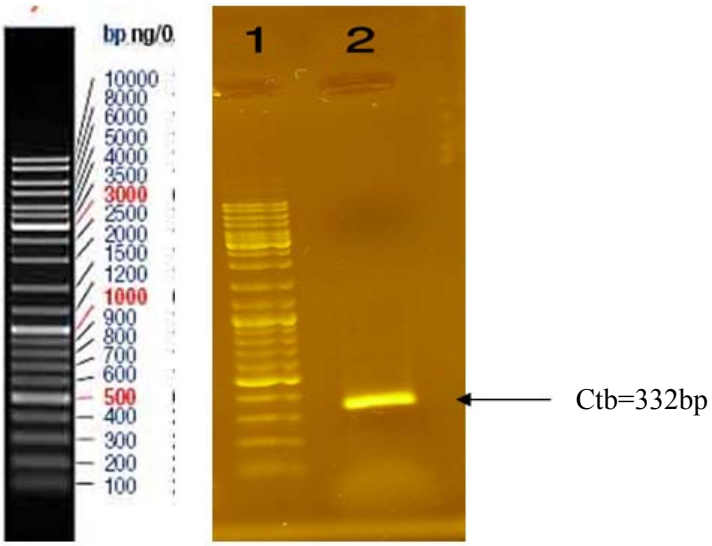

Figure 1. Agarose gel electrophoresis of PCR product amplified from ctb gene. Lane 1: DNA size marker; **Lane 2:** PCR product.

Following 5 h of induction with IPTG, a prominent band with an expected molecular weight of ~14 kDa was observed in the insoluble fraction of the bacteria containing the pET-28a-CTB plasmid; no proteins bands, however, were observed at the approximate size in either none-IPTG induction or IPTG-treated culture containing pET-28a only, revealing that the recombinant proteins were expressed in inclusion body format (Figure 3).

Purification of the CTB.
In order to purify the CTB, the crude extract was filtered and loaded onto the Ni+- resin purify column. The recombinant proteins eluted from the column were assessed by 12% SDS-PAGE. As a result, two bands, around 14 kDa, were eluted from the column (Figure 4). Western blotting analysis showed that the bands around 14 kDa reacted with goat anti-mouse anti-CTB antibody (Figure 5).

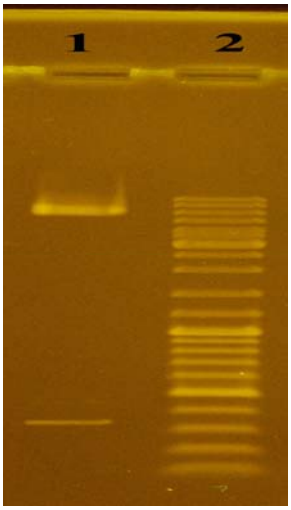

Figure 2. Restriction enzyme analysis of the constructed pET28a-ctb plasmid on 1% (w/v) agarose gel. lane 1: pET28a-ctb double digested by EcoRI and HindIII enzymes. **Lane 2:** size marker 100 bp (Fermentas).

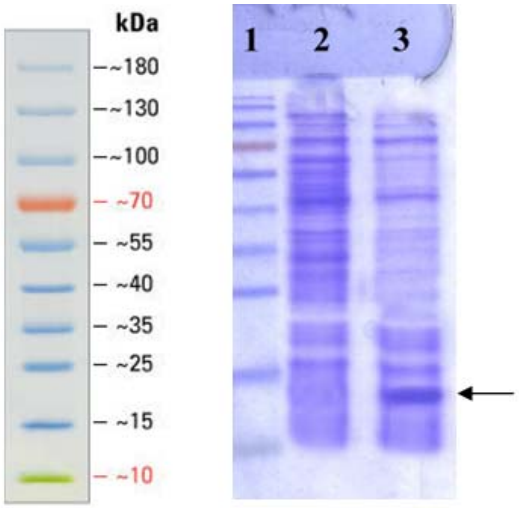

Figure 3. SDS-PAGE analysis of expression of recombinant rCTB protein. Lane 1: protein weight marker. **Lane 2:** non induced cells. **Lane 3:** The induced bacterial cells.

Purification of IgY antibody.

Water dilution method and NaCl precipitation method were utilized in order to isolate and purify IgY antibody from the egg yolk. The efficiency was estimated by SDS-PAGE under reducing conditions (Figure 6). It indicated that IgY antibody contains two major proteins, namely ~25 kDa and ~65 kDa corresponding to light and heavy chain, respectively.

Specific activity of IgY antibody against CTB determined by ELISA.

The specific activity levels of anti-CTB IgY antibody were determined by ELISA. The IgY antibody titer increased with the initial immunization and reached its peak following the third immunization. The increase in antibody titer after third

administration was significant (P<0.05). The IgY content augmented with each booster dose and remained constant after the fourth booster up to day 70, indicating the maximum protein content (figure 7). The IgY activity level in a 2000-fold dilution of the chicken serum was measured by the ELISA (OD at 492nm) using rCTB as an antigen.

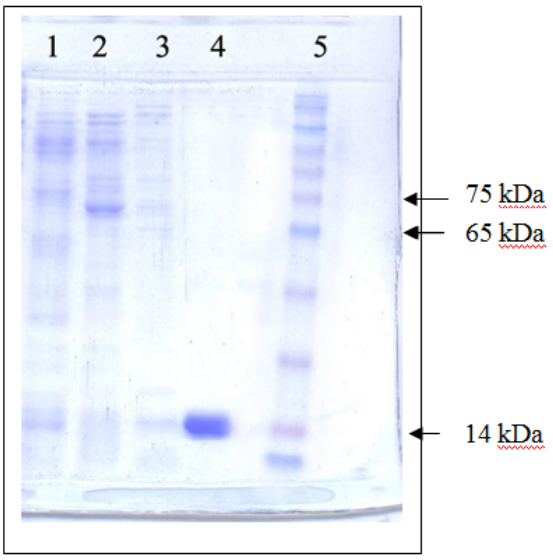

Figure 4. Purification of rCTB protein by Ni-NTA column. Lane 1: flow-through. **Lane 2:** column washed with buffer C (pH=6.8). **Lane 3:** column washed with buffer D (pH=5.9). **Lane 4:** column washed with elution buffer (pH=4.5). **Lane 5:** protein weight marker.

Infant suckling mouse challenges.

To specify the effect of anti CTB-IgY in protecting suckling mice against *V. cholerae*, the *in vivo* protective activity of anti CTB-IgY was employed at different times. Infant mice were categorized into seven categories and were separately challenged with certain doses of bacterial suspension to a lethal dose of *V. cholerae.* The inoculation of infant mice with anti CTB-IgY increased the survival rates of infant mice exposed to *V. cholerae* (Table 1).

Protection of infant mice was performed by orally administration of anti CTB-IgY. The maximum survival rates (66%) concerning challenged infant mice belonged to the third group which simultaneously inoculated yolk anti-CTB and *V. cholerae*, and the fourth group which inoculated yolk anti-CTB 1 h following the inoculation with *V. cholerae.* The survival rates of the exposed infant mice did not increase in the first group which inoculated yolk anti-CTB 2 h prior to the inoculation with *V. cholerae* (Table1). Significant difference in survival rates for groups that inoculated yolk anti-CTB after the inoculation with *V. cholera* and group that received IgY before of inoculated *V. cholerae* was observed (P<0.05).

Table 1. Protection of infant mice against lethal challenge by 4 × 10^8 to 1 × 10^9 *V. cholerae* after passive immunization with purified anti CTB-IgY. All groups were inoculated with *V. cholerae* but group number 7 was not inoculated. The seven and eight groups were passively protected with purified control nonimmune IgY.

Challenge groups	Time of inoculated anti CTB-IgY	Survivors/total Survival percent
1	2 h before of inoculated *V. cholerae*	0/6 %0
2	1 h before of inoculated *V. cholerae*	2/6 %33
3	Inoculated simultaneously	4/6 %66
4	1 h after of inoculated *V. cholerae*	4/6 %66
5	2 h after of inoculated *V. cholerae*	3/6 %50
7	Nonimmune IgY as control inoculated simultaneously	6/6 %100
8	Nonimmune IgY as control inoculated simultaneously	0/6 %0

Discussion

Cholera toxin B subunit, a candidate subunit vaccine for cholera, was expressed in *E. coli*, a valuable organism for high-level expression of proteins. This vaccine, an important protein for the prevention of cholera, has been expansively studied as an immunogen in oral vaccination.

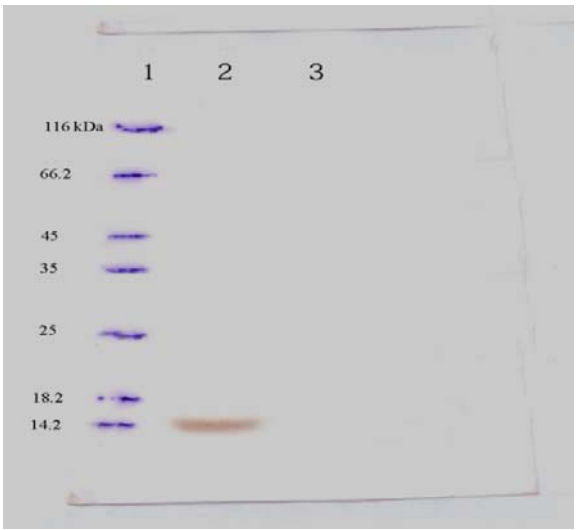

Figure 5. Western blot analysis of recombinant CTB protein. Lane 1: unstained protein ladder SM0431 (Fermentas), **Lane 2:** recombinant CTB, **Lane 3:** negative control.

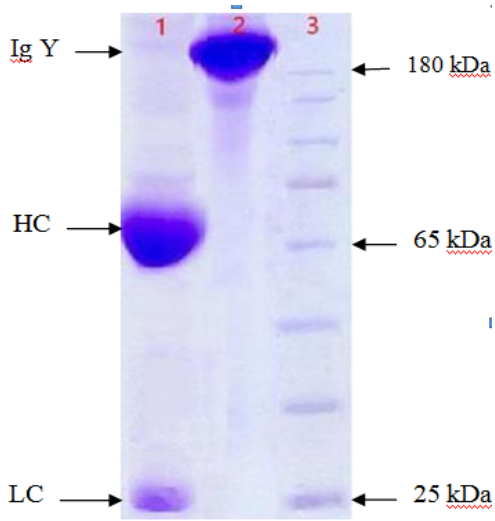

Figure 6. SDS-PAGE analysis (13 % resolving gel) of anti-CTB IgY. Lane 1: reduced IgY; **Lane 2:** non-reduced IgY; **Lane 3:** molecular weight marker.

The rCTB is expressed in a number of expression systems including insect, tobacco, tomato and potato. The production of CTB in plants and insects is limited by the fewer

stock levels. We reported and demonstrated the cloning and expression of CTB protein in *E. coli* (30). *E. coli* system remains one of the most significant organisms for recombinant protein production (31). Yolk antibodies do not activate the mammalian complement system, nor do they interact with mammalian Fc receptors which might mediate inflammatory responses in the gastrointestinal tract (22). Furthermore, eggs are normal dietary components, hence the absence of any practical risk of IgY toxic side effects. We immunized hens with rCTB. Using egg-laying hens is a cost-efficient approach to the production of large quantities of specific antibodies. Yolk antibodies are effective in preempting and controlling cholera in humans (26). The increase in the number of antibiotic resistant bacteria indicates the necessity of finding novel antibiotics. Currently, rCTB is being used in the WHO-prequalified oral cholera vaccine, Dukoral (32), and as an effectual approach to the

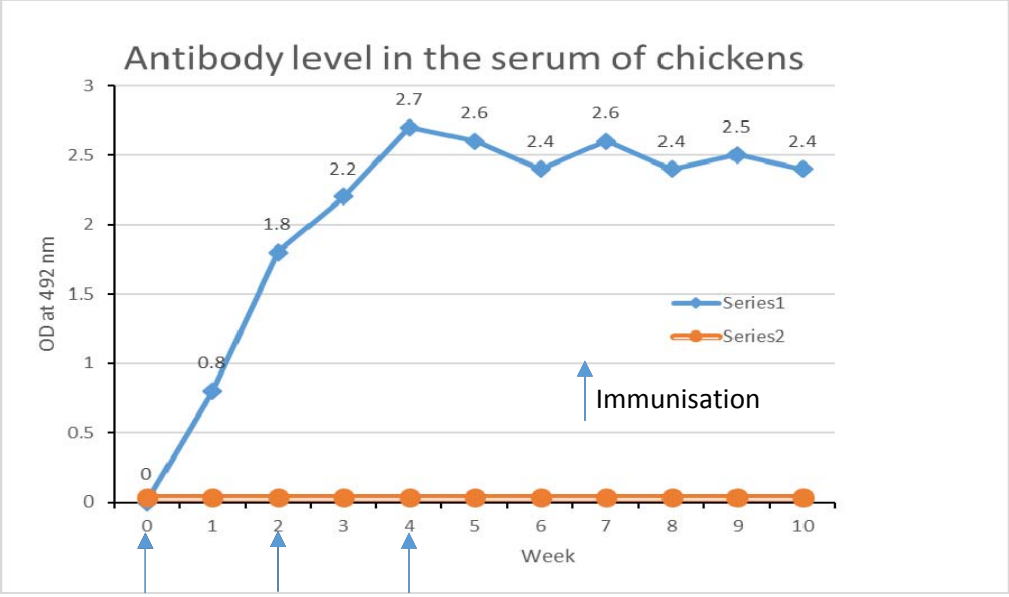

Figure 7. The titration of specific antibody raised was measured through optical density at 492 nm. Arrows indicate the week of immunization (Series1=test hens, Series2= control hens).

prevention and control of cholera since CTB-antibody obstructs the binding of CT to the intestinal mucosa and prevents its entrance in the body (33). In order to preclude the toxicity of the CT resulting from Cholera, CTB proteins have been produced in recombinant organisms. The protein band, in the present research, was slightly higher than the exact monomer CTB of the protein size owing to the 6× His-tag added to it. The His-tag fusion is the added value for the detection of recombinant protein using anti His-tag antibody. Our study showed that the utilized anti His-tag antibody was specific enough to detect the rCTB. Recombinant CTB was immunologically characterized by Western blotting using polyclonal anti-CTB antibodies through which, the protein was strongly recognized. In the present study, effort was made to generate the specific IgY against the CTB of *V. cholerae* in the serum of hens which increased following the third

week since the first immunization. So as to specify the effect of passive immunity in Yolk anti-CTB, we studied the survival rate of mice after the time of inoculation by yolk anti-CTB. The mice were exposed to a lethal dose (~100 LD50) of bacterial cells where passive immunity with yolk anti-CTB was conducted. It was demonstrated that the inoculation of yolk anti-CTB resulted in a protective effect in suckling infant mice against oral exposure to active *V. cholera* (Tables 1) which is a facile process as the suckling infant mice are susceptible to being easily infected by oral feeding only with 6 h starvation and a small dose of inoculum. The results of our study clearly indicated that suckling infant mice passively immunized with yolk anti-CTB were protected against infection when exposed to *V. cholera*. A similar protection mechanism has been observed in *V. splendidus* infections in the sea cucumber (*Apostichopus japonicus*) (34), *Enterovirus* infections in the mice (35), enterotoxigenic *Escherichia coli* infections in the piglets (36), *Staphylococcus aureus* infections in bovine mastitis (37) and *Streptococcus mutans* (38) in dental caries (39). Perez *et al.* (2001) showed that serum, along with the purified IgG and IgA from the volunteers, had a protective effect on animal models. In the research at hand, IgY was utilized for passive protection. Since using laying hens offers a cost-efficient method for the production of large quantities of specific antibodies, IgY can be easily given to people of all ages, even under serious conditions (40).

Our study showed that recombinant CTB protein is capable of being employed in the passive immunization of newborn calves against *V. cholera*. We challenged anti-CTB in suckling mouse with active bacterium in different times, but in previous works neutralizing effect of anti- CTB has be checked only in zero time . These results demonstrated that the orally-fed yolk anti-CTB effectively neutralized the cholera toxin in the gastroenteric duct, thereby binding and preventing the entry of the cholera toxin inside the cell. The binding and neutralization function appeared to be effective provided that they were applied simultaneously or 1h after the exposure to *V. cholera*. When the mice received the yolk anti-CTB through an oral passage, the bacteria and IgY passed through the gastroenteric duct and bound the yolk anti-CTB to cholera toxin, reducing the bacterial infectivity of the IgY function. The IgY specific to CTB exhibits a passive protection against cholera in mice. The first group which inoculated yolk anti-CTB 2 h before the inoculation with *V. cholera* did not increase the survival rates of the challenged infant mice; the third and fourth groups, on the other hand, showed the maximum survival rates for the exposed infant mice. The highest yolk anti-CTB functions belonged to the moment of bacteria inoculation and the following time. It seems that the lack of antibody function when used before the bacterial inoculation is related to the removal of antibodies from the digestive tract.

Our study corroborates the fact that anti CTB-IgY is effective against *V. cholera* infections in infant suckling mice when used with lethal doses (~100 LD50) of bacterial cells. Cholera was inhibited by the inoculation of anti CTB-IgY. Performing animal passive immunization by the oral administration of IgY stimulates its application in human prophylaxis. Accordingly, the anti CTB-IgY can be used for both control and prevention of cholera in humans. IgY technology could be applied as an oral supplementation for prophylaxis, and as pathogen-specific antimicrobial agents for infectious disease control.

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REFERENCES

1. Sack DA, Sack RB, Nair GB and Siddique AK. Cholera. Lancet. 2004; 363:223-233.
2. Pal P. Role of cholera toxin in *Vibrio cholerae* infection in humans - A Review. International Letters of Natural Sciences. 2014; 17:22-32.
3. Haan L, Hirst TR. Cholera toxin: a paradigm for multi-functional engagement of cellular mechanisms (Review). Mol Membr Biol. 2004; 21:77-92.
4. Faruque SM, Nair GB. *Vibrio cholerae*: Genomics and Molecular Biology. Norwich, UK: Horizon Scientific Press. 2008; 179-90.
5. Tamaki Y, Harakuni T, Yamaguchi R, Miyata T and Arakawa T. Cholera toxin B subunit pentamer reassembled from Escherichia coli inclusion bodies for use in vaccination. Vaccine. 2016; 34:1268-1274.
6. Zhang Y, Qiao X, Yu X, Chen J, et al. Enhanced soluble production of cholera toxin B subunit in *Escherichia coli* by co-expression of SKP chaperones. Protein Expr Purif. 2017; 138:1-6.
7. Hiramatsu Y, Yamamoto M, Satho T, et al. Recombinant fusion protein of cholera toxin B subunit with YVAD secreted by Lactobacillus caseiinhibits lipopolysaccharide-induced caspase-1 activation and subsequent IL-1 beta secretion in Caco-2 cells. BMC Biotechnol. 2014; 14:38.
8. Zhou Z, Dong H, Huang Y, et al. Recombinant Bacillus subtilis spores expressing cholera toxin B subunit and Helicobacter pyloriurease B confer protection against H. pylori in mice. J of Med Microbiol. 2017; 66:83-89.
9. Lebens M, Terrinoni M, Karlsson S L, et al. Construction and preclinical evaluation of mmCT, a novel mutant cholera toxin adjuvant that can be efficiently produced in genetically manipulated *Vibrio cholerae*. Vaccine. 2016; 34:2121-2128.
10. Matoba N. N-Glycosylation of Cholera Toxin B Subunit: Serendipity for Novel Plant-Made Vaccines? Front Plant Sci. 2015; 6:1132.
11. Kim MY, Kim BY, Oh SM, et al. Oral immunization of mice with transgenic rice calli expressing cholera toxin B subunit fused to consensus dengue cEDIII antigen induces antibodies to all four dengue serotypes. Plant Mol Biol. 2016; 92:347-56.
12. Soh HS, Chung HY, Lee HH. Expression and functional validation of heat labile enterotoxin B (LTB) and cholera toxin B (CTB) subunits in transgenic rice. Springerplus. 2015; 4:148.
13. Casadevall A, Dadachova E and Pirofski L. Passive antibody therapy for infectious diseases. Nat Rev Microbiol. 2004; 2:695-703.
14. Zhang WW. The use of gene-specific IgY antibodies for drug target discovery. Drug Discov Today. 2003; 8:364-71.
15. Vega C, Bok M, Chacana P, Saif L, Fernandez F and Parreno V. Egg yolk IgY: protection against rotavirus induced diarrhea and modulatory effect on the systemic and mucosal antibody responses in newborn calves. Vet Immunol Immunopathol. 2011; 142:156-69.
16. Sarker SA, Casswall TH, Juneja LR, Hoq E, Hossain I, Fuchs GJ, et al. Randomized, placebo-controlled, clinical trial of hyperimmunized chicken egg yolk immunoglobulin in children with rotavirus diarrhea. J Pediatr Gastroenterol Nutr. 2001; 32:19-25.
17. Larrie SM, Rasooli I, Mousavi-Gargari SL, Rasooli Z, Nazarian S. Passive immunization by recombinant ferric enterobactin protein (FepA) from *Escherichia coli* O157. IJM. 2013; 5:113-9.
18. Saadati M, Gorbani N, Barati B, Nazarian S. Production and Evaluation of Anti-Salmonella Typhimuruim Immunoglobulin Y Isolated from Serum and Eggs Laid. Yakhteh Medical Journal 2009; 10:261-265.
19. Gorbani N, Saadati M, Bahmani MK, Doroudian M. Production of hen egg yolk antibody against zoonotic disease (salmonellosis) and passive immunization in mice. Adv Environ Biol. 2011; 5:3710-3715.

20. Basiri H, Mousavi SL, Rasooli I, Basiri M, Yadegarynia D. Elicitation of IgY in chicken egg yolk by recombinant fragments of UreC and its efficacy against Helicobacter pylori. Adv Environ Biol. 2011; 5:3710-3715.
21. Malekshahi ZV, Mousavi-Gargari SL, Rasooli I, Ebrahimizadeh W. Treatment of Helicobacter pylori infection in mice with oral administration of egg yolk-driven anti-UreC immunoglobulin. Microb Pathog. 2011; 51:366-72.
22. Rahman S, Van Nguyen S, Icatlo FC, Umeda K, Kodama Y. Oral passive IgY-based immunotherapeutics: A novel solution for prevention and treatment of alimentary tract diseases. Hum Vaccin Immunother. 2013; 9:1039-48.
23. Harakoni T, Sugawa H, Komesu A, Tadano M, Arakawa T. Heteropentameric Cholera Toxin B Subunit Chimeric Molecules Genetically Fused to A Vaccine Antigen Iinduced Systematic and Mucosal Immune Responses: A Potential New Strategy to Target Recombinant Vaccine Antigens to Mucosal Immune Systems. Infect Immun. 2005; 73:5654-5665.
24. Green MR, Sambrook J. Molecular cloning: A laboratory manual. Cold Spring Harbor, Cold Spring Harbor Laboratory Press, NY. 2012; 3rd Edition.
25. Ladner CL, Yang J, Turner RJ and Edwards RA. Visible fluorescent detection of proteins in polyacrylamide gels without staining. Anal Biochem. 2004; 326:13-20.
26. Chalghoumi R, Beckers Y, Portetelle D, Théwis A. Hen egg yolk antibodies (IgY), production and use for passive immunization against bacterial enteric infections in chicken: a review. Biotechnol Agron Soc Environ. 2009; 13:295-308.
27. Hodek P, Trefil P, Simunek J, Hudecek J, Stiborova M. Optimized Protocol of Chicken Antibody (IgY) Purification Providing Electrophoretically Homogenous Preparations. Int J Electrochem Sci. 2013; 8:113-124.
28. Price GA, McFann K, Holmes RK. Immunization with cholera toxin B subunit induces high-level protection in the suckling mouse model of cholera. PLoS One. 2013; 8:e57269.
29. Price GA, Holmes RK. Evaluation of TcpF-A2-CTB chimera and evidence of additive protective efficacy of immunizing with TcpF and CTB in the suckling mouse model of cholera. PLoS One. 2012; 7:e42434.
30. Gong Z, Long X, Pan L, Le Y, Liu Q, Wang S, et al. Cloning, expression, purification and characterization of the cholera toxin B subunit and triple glutamic acid decarboxylase epitopes fusion protein in *Escherichia coli*. Protein Expr Purif. 2009; 66:191-197.
31. Lewis G, Taylor IW, Nienow AW and Hewitt CJ. The application of multi-parameter flow cytometry to the study of recombinant Escherichia coli batch fermentation processes. J Ind Microbiol Biotech. 2004; 31:311-322.
32. WHO. Cholera vaccines: WHO position paper. Wkly Epidemiol Rec. 2010; 85:117–28.
33. Lopez AL, Antoinette Gonzales ML, Aldaba JG and GB Nair. Killed oral cholera vaccines: history, development and implementation challenges. Ther Adv Vaccines. 2014; 2:123-136.
34. Li X, Jing K, Wang X, Li Y, Zhang M, Li Z, et al. Protective effects of chicken egg yolk antibody (IgY) against experimental Vibrio splendidus infection in the sea cucumber (Apostichopus japonicus). Fish Shellfish Immunol. 2016; 48:105-11.
35. Liou JF, Chang CW, Tailiu J, Yu CK, Lei HY, Chen LR, et al. Passive protection effect of chicken egg yolk immunoglobulins on enterovirus 71 infected mice. Vaccine. 2010; 28:8189-8196.
36. Alustiza F, Bellingeri R, Picco N, Motta C, Grosso MC. IgY against enterotoxigenic Escherichia coli administered by hydrogel-carbon nanotubes composites to prevent neonatal diarrhoea in experimentally challenged piglets. Vaccine. 2016; 34:3291-3297.
37. Mahenthiran R, Naveen S, Diraviyam T, Ravikumar K and Michael A. Generation, purification and neutralization potential of chicken egg yolk antibodies (IgY) against mastitis causing E. coli and Staphylococcus aureus. Int J Pharm Bio Sci. 2013; 4:687-696.
38. Handajani J, Narissi DH, Azis M, Rahmawati AS. Effects of immunoglobulin Y(IgY) serum against plaque bacteria. Afr J Microbiol Res. 2016; 10:1668-1674.
39. Barati B, Ebrahimi F and Nazarian S. Egg yolk antibodies for disease prevention. J Bacteriol Mycol Open Access. 2016; 3:0058.
40. Perez JL, Garacia L, Talavera A, Oliva R, Valmaseda T, et al. Passive protection of serum from volunteers inoculated with attenuated strain 638 of *Vibrio cholerae* O1 in animal models. Vaccine. 2001; 19:376-384.